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Production of grape marc kombucha: Valorization of a wine by-product

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ABSTRACT

The revalorization of by-products is a priority for sustainable winemaking. This study explores the potential of white grape marc as a raw material for producing a kombucha-style beverage. We compared the fermentation processes of traditional tea kombuchas (green and black) and grape marc kombucha (Grape-K), all fermented with a homemade Symbiotic Culture of Bacteria and Yeast (SCOBY) cultured in grape marc infusion. The results demonstrated comparable acid production across all variants, with Grape-K showing enhanced sugar consumption. The microbial community was primarily composed by *Brettanomyces bruxellensis* (yeast), *Oenococcus oeni* and *Liquorilactobacillus ghanensis* (lactic acid bacteria), and *Komagataeibacter intermedius* (acetic acid bacteria). Although Grape-K contained lower levels of phenolic compounds and higher acetic acid concentrations than tea-based kombuchas, it exhibited a significant increase in antioxidant activity post-fermentation. Using Muscat grape marcs, known for their enhanced floral notes, improved the organoleptic profile of the beverage. The balance of volatile acidity with other aromatic compounds in Grape-K highlights the potential of grape marc, especially from aromatic white varieties, as a promising raw material for creating kombuchas with distinct and appealing aromatic profiles.

1. Introduction

Kombucha, a fermented tea beverage with origins traced back to ancient China (about 220 B.C.), has gained widespread popularity due to its perceived health benefits and unique sensory profile (Jayabalan et al., 2014). Traditionally, kombucha is produced by fermenting sweetened green or black tea with a symbiotic culture of bacteria and yeast (SCOBY) also known as tea fungus.

This SCOBY gradually breaks down sugar and leads to a metabolic cascade that produces CO₂ and an acidic, slightly alcoholic beverage (Chakravorty et al., 2016). The SCOBY is usually described as composed by acetic acid bacteria (AAB, e.g., *Acetobacter*, *Gluconobacter*, and *Komagataeibacter* spp.), yeasts, such as *Saccharomyces*, *Debaryomyces*, *Brettanomyces* and *Kluyveromyces* spp. and lactic acid bacteria (LAB, such as *Lactobacillus* and *Oenococcus* spp.) (Antolak et al., 2021; Coton et al., 2017; Gaggia et al., 2019; Tran et al., 2020). However, recent metagenomics analysis of the microbial community of the kombucha has revealed a far more complex consortium of above 200 species across 34 genera (Kaashyap et al., 2021).

The fermentation process, which is normally set between 7 and 10 days, not only provides the characteristic tangy flavor but also enhances

the drink's bioactive properties, including its antioxidant capacity (Villarreal-Soto et al., 2018). During the kombucha fermentation process, the components contained in the tea infusion and the newly formed compounds by the SCOBY would contribute to the unique flavor, various biological activities as well as health-promoting properties of fermented kombucha (Bhattacharya et al., 2020; Liu et al., 2022; Vitas et al., 2018). Therefore, the chemical composition and bioactivity of kombucha might change vastly for different types of tea or other substrates used (Cardoso et al., 2020; Villarreal-Soto et al., 2019).

In recent years, there has been a growing interest in developing novel kombucha analogs using various substrates, driven by both the desire to diversify flavors and functional products and the need to utilize sustainable raw materials (Cardoso et al., 2020; Leonarski et al., 2022; Liu et al., 2022; Morales et al., 2023). To date, a great variety of substrates have been tested, such as different plant leaves, fruits, herbaceous plants, dairy products, grains, and agro-industrial by-products (reviewed in Su et al., 2023).

Grapes are one of the most cultivated fruit crops worldwide with approximately 77 Mt produced in 2020, 57% of which was used for winemaking (OIV, 2022). Grape marc, the solid residue remaining after grape pressing in winemaking, accounts for 20–30% of the total grape

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weight and must be treated, disposed of, or reused properly to reduce its pollution load (Corbin et al., 2015). It has been previously used as a soil amendment and source of natural phytochemicals. The composition of grape marc can vary depending on processing techniques and grape variety, but typically, the moisture content ranges from 50 to 60% of the fresh marc and residual sugars constitute 3–8%. Regarding grape marc dry weight composition, phenolic compounds can account for 15–30 g/kg, organic acids are typically between 4 and 6%, proteins range from 5 to 10%, free amino acids are scarce accounting for 1–3%, lipids are mainly in the seed representing 6–12% in the marc, minerals can make up to 4–7% and, finally, the lignocellulosic material is quite abundant (lignin, cellulose and hemicellulose) ranging from 40 to 57% (Bustamante et al., 2008; Fontana et al., 2013). Rich in polyphenols, fibers, and other bioactive compounds, grape marc is a valuable but underutilized by-product of the wine industry (Gómez-Brandón et al., 2019). Thus, grape marc presents a promising alternative substrate to produce a kombucha analog. Previously, grape marc has been fermented for the growth of lactic acid bacteria and bifidobacteria with the perspective of producing a functional ingredient having antioxidant activity (Campanella et al., 2017). Also, red grape juice and berries have been used to produce kombucha analogs and their antioxidant activities have been investigated (Ayed et al., 2017; Morales et al., 2023). Winery effluents during red wine production have been fermented by a kombucha SCOBY showing that the resulting kombucha was rich in organic acids, vitamin C, total polyphenols, and strong antioxidant activity (Vitas et al., 2018). Finally, grape pomace from a blend of red grape varieties has been used for the first time to produce kombucha (Barakat et al., 2024).

The winery by-product grape marc from white varieties has not yet been investigated as a substrate for kombucha fermentation. Water-soluble carbohydrates in white marc comprise approximately one-third of its dry weight and 70% of the total carbohydrate content in contrast to red grape marc, which contains a much lower percentage of soluble carbohydrates (Corbin et al., 2015). This high concentration of soluble carbohydrates makes white marc particularly appealing for kombucha production, as it reduces the need for added sugars. Additionally, during the production of white wine, grape marc is immediately discarded and not used for soaking with the must to extract colour, as it happens with red wine.

The objective of this study was to produce a functional beverage using grape marc as the raw material and a commercial unpasteurized kombucha as starter of the fermentation. We aimed to comprehensively analyze the characteristics of the fermented beverage in comparison with traditional green and black tea kombucha. This involved the identification of the microbial community dynamics, physicochemical parameters, antioxidant capacity, and sensory attributes of the grape marc kombucha.

By exploring the feasibility of using grape marc in kombucha production, this study aims to contribute to the development of innovative, sustainable beverage options and to provide a value-added application for grape marc. The findings could have significant implications for both the beverage industry and the sustainable management of winemaking by-products.

2. Materials and methods

2.1. Raw materials and starter culture

Green Sencha tea and black Ceylan tea leaves were purchased from the local store “Tea house” at Tarragona (Spain), together with the unpasteurized kombucha of the commercial brand “Mun” (Mun Ferments SL, Mataró, Spain), that was used at the first place as starter culture.

Two different experiments were designed, and different grape marcs were used. To test if a kombucha-like drink could be obtained out of grape marc, 1 kg of grapes of the varietal Rosetti was purchased at a local fruit store from Tarragona. These grapes were pressed and filtered

with a cotton fabric discarding the juice while the skin, seeds, and stem (grape marc or pomace) were saved for grape kombucha preparation. For the second experiment, grape marc was collected during the pressing of Muscat of Alexandria grapes at Mas dels Frares, the experimental cellar of the University Rovira i Virgili. The fresh grape marc was transported to the laboratory and used within the same day of the grape pressing.

2.2. Kombucha fermentation and monitoring

For the first experiment, three kombucha variants were prepared: one with green leaves, another with black tea leaves, and the third using fresh grape marc (Fig. 1). The tea kombuchas were prepared traditionally. Briefly, tea leaves (10 g/L) were added to 0.8 L of sterile hot water without hypochlorite (85 °C) together with 60 g/L of white cane sugar (Azucarera, Madrid, Spain). The grape marc kombucha was prepared similarly to the others, but with 200 g/L of grape marc replacing the tea leaves in hot water, supplemented with 10 g/L of cane sugar. The reason to include less sugar was that residual monosaccharides retained at the grape marc are expected to be 10–17 % w/w being 5–6 % w/w glucose depending on the grape varietal (Corbin et al., 2015). The mixtures were stirred and left to brew for 10 min and then filtered through sterile cotton fabric leaving approximately 0.75 L of each infusion. When the filtrates were cooled down to room temperature, 150 mL/L of commercial non-pasteurized kombucha was added as starter to each filtrate and the 1 L jars were covered with a double layer of cotton fabric adjusted to the top with rubber bands. After 21st days of static, room-temperature incubation (25–28 °C) in darkness, a thick (0.8–1 cm wide) biofilm was formed at the surface of the three kombuchas. The final pH and acidity were measured and both the liquid and biofilm of the grape kombucha were stored at 4 °C. The differences between the two kombucha starters, the one commercial and the one used to produce the SCOBY can be found in Suppl. Table 1.

For the preparation of the second experiment (Fig. 1), 2 L duplicates of each kombucha type were prepared in the same way that the first ones. However, the starter or inoculant of the second round of kombucha was 150 mL of liquid and a portion of the biofilm that resulted from the first grape marc kombucha. The density, pH, and total acidity were measured daily, while microscopy, CFU, and physicochemical measurements were done on days 0 and 7. After 7 days of fermentation, the biofilms were removed and duplicates of each kombucha were pooled avoiding the bottom deposit of yeast and kept at 4 °C until sensorial test was done. For the rest of the analysis, samples were frozen at –20 °C and centrifuged before chemical analysis (3000×g for 10 min).

2.3. Microbial identification

The populations of yeasts and bacteria during fermentation were determined by both microscopy counting and by plating successive decimal dilutions of samples on YPD and WLN agar for yeasts and MRSmf and GY-C agar for bacteria. The composition of the media was 2 % glucose, 2 % peptone, and 1 % yeast extract for the YPD medium; 7.5 % of Wallerstein Laboratory Nutrient (Oxoid, England) for the WLN medium; 5.5 % of MRS, 0.5 % fructose, 0.6 % D, L-malic acid, 0.05 % cysteine for MRSmf medium; 5 % glucose, 1 % yeast extract, and 1 % CaCO₃ for GY-C medium. Both MRSmf and GY-C were supplemented with 100 mg/L of nystatin (Panreac Química SLU, Castellar del Vallès, Spain) to avoid fungal growth after being autoclaved. Before being inoculated, 40 µL of 100 mg/L of a sodic azide (G BioSciences, St. Louis MO, USA) solution was spread onto MRSmf plates to avoid AAB. All the media were solidified with the addition of 1.5 % agar and all the reagents were purchased from Difco (Detroit, MI, USA) except specified otherwise. CFU counting was performed after 24 h of incubation at 28 °C for YPD and WLN and after 72 h for GY-C and MRSmf.

Differences in morphology and color of the colonies were used to select the yeast and bacteria for observation under the microscope and

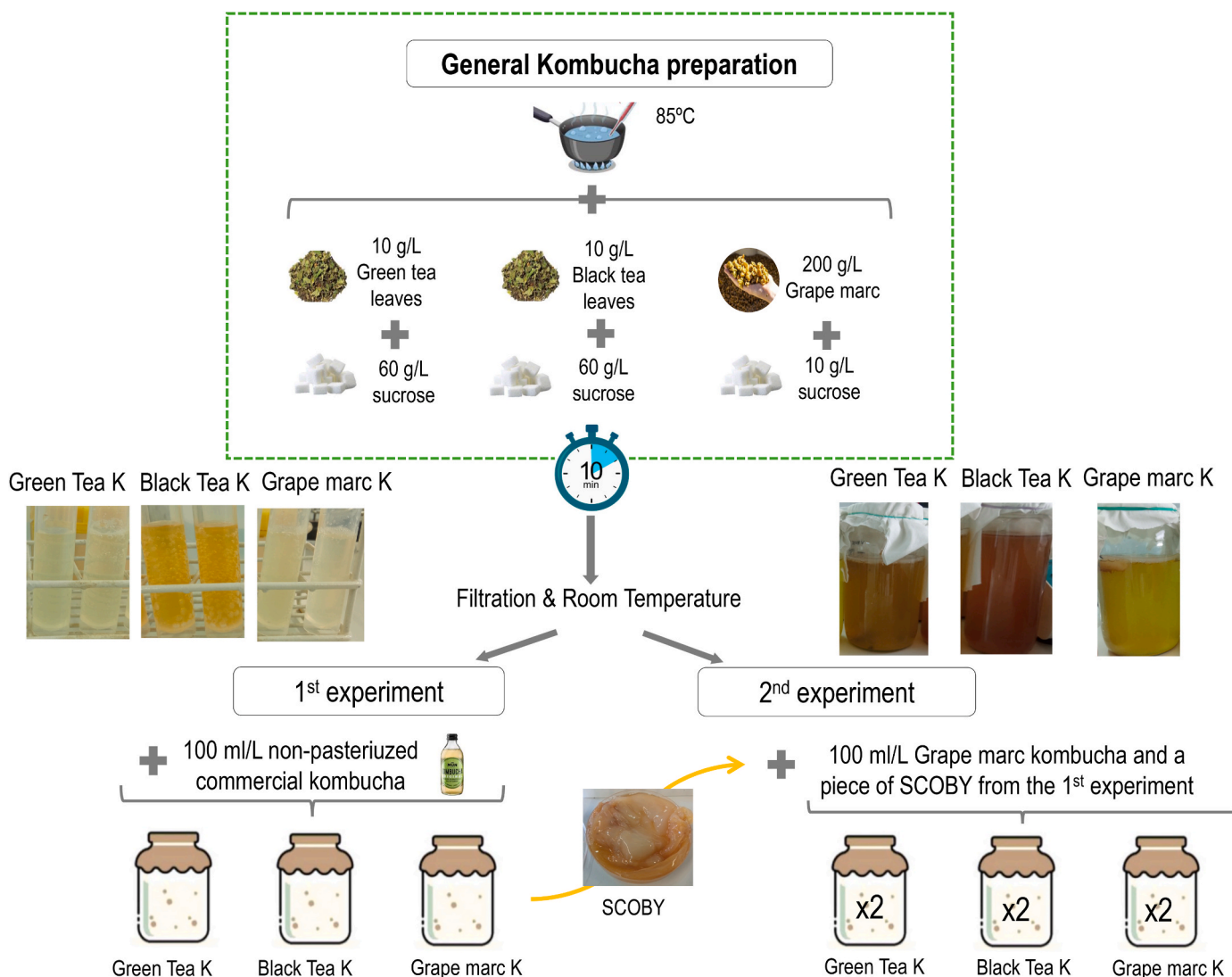


Fig. 1. Schematic representation of the experimental design.

molecular identification based on DNA.

To obtain the genomic DNA of the colonies, they were suspended in 100 μ L of sterile water and subjected to three 5-min cycles of 95 °C and ice incubations. Then, the samples were centrifuged at 5000 \times g for 3 min and the supernatant was used directly as a template for the PCR amplifications. We did not perform any protein precipitation, and DNA was not quantified or qualified, as it was not necessary for our application.

The isolated yeasts were identified based on the RFLPs of the PCR-amplified ITS-5.8S rDNA region as described by (Esteve-Zarzoso et al., 1999). The RFLP patterns of the yeast isolates were compared through the database of www.yeast-id.com (<http://www.yeast-id.org/english/index.html>) and grouped to known species. Yeast identification was confirmed by sequencing the PCR-amplified D1/D2 domain of the 26S rDNA of representative yeast colonies isolated from each sample.

The PCR amplification and sequencing of the 16S rDNA region identified the isolated bacteria using the primers pA and pH (Edwards et al., 1989; Rodas et al., 2003). Primer sequences and PCR conditions from the present study are indicated in the [Supplementary Table 2](#).

Identification was considered appropriate with similarities higher than 99%. The sequencing was performed by Macrogen (Madrid, Spain). The sequences have been deposited at GenBank (<https://submit.ncbi.nlm.nih.gov/about/genbank/>) under the accession numbers PQ363626-PQ363640 (bacterial isolates) and PQ365989-PQ365995 (yeast isolates).

2.4. Chemical characterization of kombucha

Kombuchas were characterized chemically at 0 and 7 days of fermentation, except for the density, pH, and acidity that were monitored daily. Chemical density was measured using an electronic densitometer (Densito 30PX Portable Density Meter; Mettler Toledo, Spain). The pH values were obtained by a Crison micro pH 2002 pH-meter (Hach Lange Spain, l'Hospitalet, Spain). Total acidity was determined by titration with 0.1N NaOH (Panreac Química SLU, Castellar del Vallès, Spain) and expressed as g/L of acetic acid following the indications of OIV methods (OIV, 2015).

The concentration of reductive sugars (glucose and fructose), acetic acid, ammonia, citric acid, L-malic acid, D- and L-lactic acids, and PAN (primary amino nitrogen) were determined by enzymatic methods using Y15 ANALYZER (Biosystems, Barcelona, Spain).

Sucrose was determined by the Enzytec™ Liquid Sucrose/D-Glucose commercial kit (R-Biopharm AG, Darmstadt, Germany) according to the manufacturer's instructions.

The total anthocyanin concentration was determined by the discoloration of wines with sodium metabisulphite (Fisher Scientific, Madrid, Spain) (Balmaseda et al., 2021).

Ethanol and tartaric acid were determined in an Agilent 1100 HPLC (Agilent Technologies, Germany). Samples were centrifugated at 13,000 \times g for 5 min and the supernatant was filtered with 0.22 μ m pore

filters before injection (Agilent Technologies) (Quirós et al., 2010). The HPLC had coupled a Hi-Plex H (300 mm × 7.7 mm) column inside a 1260 MCT (Infinity II Multicolumn Thermostat). The column conditions involved maintaining a temperature of 60 °C for 30 min, with the mobile phase at 5 mM H₂SO₄ flowing at a 0.6 mL/min rate. Additionally, the chromatograph was equipped with two detectors: an MWC detector (G1365B multi-wavelength detector) and RID detector (1260 Infinity II refractive index detector) (Agilent Technologies, Germany).

The total phenolic content (TPC) was measured using the Folin Ciocalteu reagent. A standard curve was generated by combining 100 µL of gallic acid standard solution (ranging from 75 to 1000 mg/L) with 500 µL of Folin-Ciocalteu reagent and 2 mL of 20 % (w/v) Na₂CO₃ solution, in a reaction mixture with total volume of 10 mL. Kombucha samples were analyzed in the same way, substituting gallic acid with the respective kombucha samples. Reaction tubes were incubated in darkness at RT for 30 min. The reduction of phosphotungstate-phosphomolybdate heteropolyacid salts was measured at 760 nm. A blank sample was prepared by replacing the 100 µL of sample with distilled water. The results are expressed as mg of gallic acid equivalents/L (mg GAE/L).

2.5. Antioxidant capacity measured as DPPH scavenging activity

The antioxidant capacity of the produced kombuchas was measured using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) as described by (Villarreal-Soto et al., 2019). Briefly, 100 µL of kombucha (2-fold serial dilutions; from direct to 1/2048 dilution) were mixed with 500 µL of a freshly prepared DPPH ethanolic solution (7.5 mg/100 mL). The mixture was vortexed and incubated in darkness at RT for 1 h. Then, absorbance at 520 nm was measured after vortexing and centrifuging the mixture at 3000×g for 10 min. The antioxidant capacity was expressed as the inhibition exhibited in the mixture reaction according to the following equation:

$$\text{Inhibition (\%)} = \frac{Abs_0 - Abs_{\text{sample}}}{Abs_0} \cdot 100 \quad \text{Eq. 1}$$

where Abs₀ is the absorbance of the DPPH ethanolic solution using 100 µL of distilled water as sample.

The resulting inhibition percentages were adjusted to a sigmoidal curve to obtain their equation, with the following form:

$$Y = \frac{a}{(c + e^{(-b \cdot x)})} \quad \text{Eq. 2}$$

where Y is the inhibition percentage, x is the concentration of kombucha in mL/L of reaction, and a, b, and c are the constants of the sigmoidal model.

The IC₅₀ value was interpolated, which represents the concentration of kombucha (mL/L of reaction) needed to inhibit half of the total DPPH concentration in the reaction mixture.

2.6. Sensory analysis

Sensory analysis was performed with the produced green, black, and grape marc kombuchas (Green-K, Black-K, and Grape-K, respectively). Duplicates were blended to simplify the analysis. Kombuchas were stored in the fermentation container at 4 °C until the tasting. The day before the tasting, the three kombuchas were filtered with filtration paper to avoid the presence of any solid SCOBY rests. Kombuchas were evaluated by 15 tasters, considered as experts in wine tasting, from the Oenology Faculty of Rovira i Virgili University.

First, the experts were given 26 mL of kombucha in dark glasses to avoid subjectivity due to the color of the samples. Glasses were randomly numbered with 3-digit codes. Kombuchas were served anonymously according to a Latin square of Williams design to avoid the range and carry-over effect. Triangle tests were performed to evaluate differences between the produced kombuchas. In addition, tasters were

asked to write down their preferences in each sequence.

Second, experts were asked to do a descriptive test of the three kombuchas in clear ISO glasses. The descriptive test emphasized (i) the visual characteristics, (ii) aroma and (iii) flavor attributes: (i) color intensity, and transparency, (ii) aromatic intensity, floral attribute, citric attribute, and volatile acidity, and (iii) acidity, sweetness, and bitterness. In addition, experts ranked the kombuchas based on their overall quality. Tasters had to score in a structured scale from 1 (no detection) to 5 (the highest) the intensity of each attribute.

2.7. Statistical analysis

The data of duplicate experiments were expressed as the mean and standard deviation. Statistical analysis was performed using the software XLSTAT version 2020.2.3.65345 (Addinsoft, Paris, France). To test for differences between samples, a one-way ANOVA was performed using Tukey's honest significant difference post-hoc test at a p-value of 0.05. The same software and the statistical test were used to identify significant differences in the data of the triangle test and the mean of scores of the descriptive attributes during the sensory analysis.

3. Results and discussion

3.1. Kombucha fermentation process

Kombucha fermentation process involves the consumption of sugar, which leads to a decrease in the initial density, and the production of high concentrations of acids. This accumulation of acids increases total acidity during fermentation and subsequently lowers the pH value. In the first experiment, a commercial starter of unpasteurized green tea kombucha with an initial density of 1007 g/L and a pH of 2.70 was used. For the second experiment, a fermented grape marc starter with a similar density (1009 g/L) but a slightly higher pH of 3.10 was utilized.

Fig. 2 illustrates the fermentation performance of the three kombuchas produced during the second experiment, in terms of density and pH decrease. The two tea infusions – green and black tea – started with similar initial density (about 1020 g/L) and pH values (about 5.50). The density decrease was minimal in both tea fermentations. In contrast, the pH decreased dramatically on the first day of fermentation, from above 5.00 to values below 3.50 for both types of kombucha (Fig. 2).

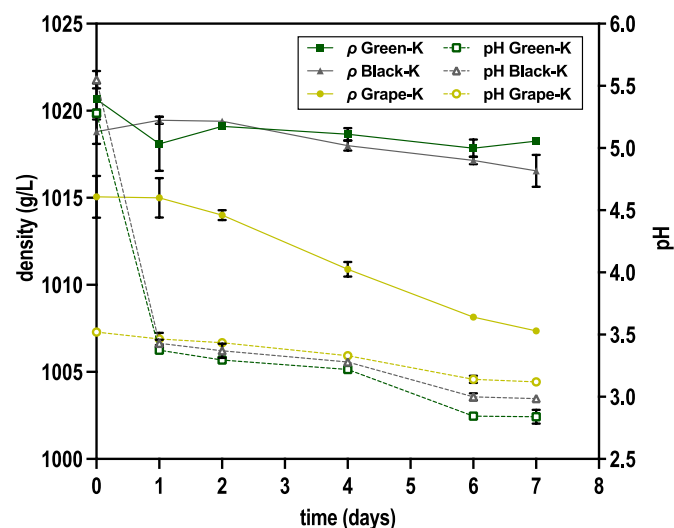


Fig. 2. Evolution of density and pH during the fermentative process of the three produced kombuchas: Green-K, Black-K, and Grape-K means green tea kombucha, black tea kombucha, and grape marc kombucha, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Grape marc infusion had a different starting point, and its fermentation performance was slightly different. First, the initial density was about 5 units lower and then decreased more than the other two infusions (Fig. 2). Second, the initial pH of grape marc infusion was lower, according to its origin since grapes are known to have a high organic acid concentration (Soyer et al., 2003). Regardless of the raw material, the final pH of all three kombuchas was similar (Fig. 2). These pH values, about 3.00, closely match that of the grape marc starter used. The resulting pH values are consistent with previous studies on fermented green and black teas (Wang et al., 2022) and grape juice (Ayed et al., 2017), indicating a safe pH range for human consumption, between 2.50 and 4.20.

3.2. Kombucha characterization

3.2.1. Microbial community

Understanding the microbial community is crucial, as it plays a pivotal role in the fermentation process, influencing both the safety and the sensory qualities of the final product (Wang et al., 2023).

The quantification of cells under the microscope of samples from the starter and the three types of kombucha were similar (Table 1). A volume of 100 mL/L of starter was used for the preparation of kombucha in the second experiment, hence inoculating 5×10^5 cells/mL. The total number of culturable yeasts, LAB, and AAB were determined for each kombucha of the second experiment after 7 days of fermentation denoting an increase in population as a consequence of the fermentative process (Table 1). The CFU/mL recovered in the YPD medium generally agreed with the cells quantified under the microscope (Table 1). The exception was the black tea kombucha that obtained one order of magnitude higher of CFU/mL in YPD. The LAB detected in the MRSmf medium were between 5×10^3 and 7×10^4 CFU/mL in all cases and the AAB were between 5×10^4 and 1×10^5 CFU/mL. These numbers of microorganisms generally agree with previous studies in green or black tea kombucha (Coton et al., 2017; Kaashyap et al., 2021).

A total of 48 yeast colonies from YPD and WLN media and 21 bacterial colonies from MRSmf and 10 from GY-C were isolated. A selection of these colonies based on their morphology and origin of the sample was used for PCR amplification and sequencing. The only yeast identified in the three types of kombucha from the PCR products sequenced was *Brettanomyces bruxellensis*. Within the LAB bacteria isolated from MRSmf medium we identified mainly *Oenococcus oeni* (80.6%) and *Liquorilactobacillus ghanensis* (19.4%). *O. oeni* has been the most abundant LAB detected in commercial green and black kombucha and strongly associated with green kombucha (Coton et al., 2017). We detected *O. oeni* both in Green-K and Grape-K, but not in Black-K. The only AAB isolated was *Komagataeibacter intermedius*. These genera were the most frequently isolated and also detected by molecular analysis in previous kombucha studies (Coton et al., 2017; Villarreal-Soto et al., 2020).

Table 1

Microbial composition of the commercial non-pasteurized kombucha (starter), the non-fermented kombucha at day 0, and the three different produced kombuchas after 7 days of fermentation.

	Starter	NF-kombucha	Green-K	Black-K	Grape-K
Microscopy (Log cell/mL)	6.70 ± 0.3	4.7 ± 0.3	6.30 ± 0.7	6.60 ± 0.6	6.40 ± 0.6
YPD (Log CFU/mL)	6.65 ± 0.8	4.65 ± 0.8	6.30 ± 0.3	7.18 ± 1.1	6.67 ± 0.5
MRS (Log CFU/mL)	4.85 ± 0.5	2.85 ± 0.5	3.78 ± 0.7	3.70 ± 0.3	4.70 ± 0.5
GY-C (log CFU/mL)	ND	ND	3.00 ± 0.4	5.00 ± 0.2	3.70 ± 0.1
WLN (Log CFU/mL)	ND	ND	6.30 ± 0.4	4.70 ± 0.4	6.30 ± 0.8

ND: not determined. NF: non-fermented kombucha infusion at day 0.

3.2.2. Chemical composition

Kombuchas before (non-fermented, NF) and after seven days of fermentation (fermented, K) were chemically characterized (Table 2). To allow kombucha fermentation, the initial kombuchas' were supplemented with commercial sucrose for about 60 g/L in the case of tea kombuchas, and 10 g/L in the case of grape marc kombucha. The concentration of added sugar to Green-K and Black-K was within the normal range used for kombucha production which is 5–10% (Jayabalan et al., 2014). The difference in sucrose addition to Grape-K responded to an increased concentration of reductive sugars (glucose and fructose) coming from the grape marc itself (Table 2). In all cases, there was a general reduction in sucrose concentration due to the fermentation process. This sucrose was hydrolyzed into glucose and fructose, which were higher in the case of Green-K and Black-K (Table 2). Notably, Grape-K had similar reductive sugar concentration to Green-K and Black-K, but considerably lower reductive sugars than the Grape-NF as result of the consumption of the initial quantity naturally present in the grape marc. However, Grape-K resulted in lower sucrose concentration than the other two types of kombucha (Table 2), representing a healthier

Table 2

Chemical composition of the non-fermented and fermented kombuchas during the second experiment. Green, black and grape means green tea, black tea, and grape marc, respectively. NF: non fermented; K: kombucha.

	Green-NF	Black-NF	Grape-NF	Green-K	Black-K	Grape-K
Sucrose (g/L)	62.69 ± 1.80 ^b	62.40 ± 0.23 ^b	10.88 ± 0.67 ^a	17.20 ± 1.46 ^{ab}	17.14 ± 0.46 ^{ab}	5.78 ± 0.02 ^a
Reductive sugars ^a (g/L)	0.35 ± 0.07 ^a	0.70 ± 0.14 ^a	51.15 ± 2.62 ^c	31.20 ± 2.26 ^b	20.10 ± 8.06 ^b	26.35 ± 0.21 ^b
Citric acid (mg/L)	79 ± 11 ^b	96 ± 8 ^b	24 ± 12 ^a	77 ± 5 ^b	76 ± 15 ^b	7 ± 1 ^a
L-malic acid (g/L)	<0.05	<0.05	0.28 ± 0.01 ^b	<0.05	<0.05	0.07 ± 0.01 ^a
L-lactic acid (g/L)	0.10 ± 0.01 ^{ab}	0.07 ± 0.01 ^a	<0.05	0.12 ± 0.04 ^{ab}	0.11 ± 0.05 ^{ab}	0.19 ± 0.01 ^b
D-lactic acid (g/L)	<0.05	<0.05	<0.05	0.09 ± 0.01	0.12 ± 0.02	0.15 ± 0.04
Acetic acid (g/L)	<0.10	<0.10	<0.10	2.30 ± 0.71 ^b	2.20 ± 0.28 ^a	3.10 ± 0.14 ^c
Tartaric Acid (g/L)	<0.25	<0.25	0.81 ± 0.04 ^a	<0.25	<0.25	0.98 ± 0.06 ^a
Total acidity (g/L)	0.22 ± 0.03 ^a	0.22 ± 0.03 ^a	0.32 ± 0.11 ^a	3.40 ± 0.01 ^c	2.90 ± 0.14 ^b	3.90 ± 0.14 ^d
Ethanol (% vol/vol)	<0.20	<0.20	<0.20	0.22 ± 0.02 ^a	0.28 ± 0.01 ^b	0.90 ± 0.23 ^c
PAN ^b (mg N/L)	19.0 ± 2.8 ^{ab}	24.5 ± 5.0 ^b	11.5 ± 0.7 ^a	<10	<10	<10
Ammonia (mg N/L)	21.0 ± 5.7 ^{abc}	26.5 ± 3.5 ^c	20.5 ± 5.0 ^{bc}	16.0 ± 9.9 ^a	16.5 ± 0.7 ^{abc}	13.0 ± 2.8 ^{ab}
Phenolic acids (mg GAE/L)	639 ± 17 ^b	919 ± 110 ^c	120 ± 1 ^a	644 ± 47 ^b	877 ± 85 ^{bc}	104 ± 27 ^a
Anthocyanins (mg/L)	6.1 ± 2.5 ^b	3.9 ± 0.6 ^a	2.6 ± 0.1 ^a	n.d.	3.5 ± 1.2 ^a	3.1 ± 0.6 ^a
Maximal antioxidant capacity (%)	91 ± 1 ^b	94 ± 2 ^{bc}	61 ± 1 ^a	96 ± 1 ^c	96 ± 1 ^c	92 ± 1 ^{bc}
Inhibition IC ₅₀ (mL kombucha/L)	1.86 ^a	1.83 ^a	124.02 ^c	2.33 ^a	1.92 ^a	51.32 ^b

^a Reductive sugars: glucose + fructose.

^b PAN: primary amino nitrogen. Superscripts indicate significant differences at $p \leq 0.05$ according to a Tukey post-hoc comparison test. No letter means no significant difference ($p \leq 0.05$). n.d.: not detected.

alternative in this sense. The primary microorganism consuming sugar are yeasts, but also LAB and AAB as *Komagataibacter* consume different sugar fonts to produce organic acids. Longer fermentations could result in lower residual sugar at the produced beverage but higher acetic acid and ethanol concentrations.

In addition, some organic acids were quantified. Citric acid concentration was low in both fermented and non-fermented kombuchas but decreased due to the fermentation. Yeast cells can consume citric acid under certain conditions, to replenish tricarboxylic cycle intermediates, if the yeast requires acetyl-CoA for the synthesis of fatty acids and sterols or to regulate pH and maintain the redox balance (Mendes Ferreira & Mendes-Faia, 2020). Furthermore, certain LAB as *Lactobacillus* and *Oenococcus* can utilize citric acid as a carbon source converting it to lactic acid to support energy production and growth (Mendes Ferreira & Mendes-Faia, 2020). This dual action of yeast and LAB contributes to the reduction of citric acid during fermentation. Malic acid was detected just in grape marc modalities (Table 2). The reduction of L-malic acid is related with malolactic fermentation that can be performed by LAB in fermented beverages. Even so, the total lactic acid observed in all three kombuchas was similar, probably due to a similar sugar fermentative performance of LAB population (Table 2). Acetic acid, which is the major organic acid compound found in kombucha (Tran et al., 2020), went from not detectable in the non-fermented kombuchas to about 3 g/L in the fermented ones (Table 2). The increase of this compound is directly related to the metabolism of all the microorganisms involved in the SCOBY, but mainly to AAB (Tran et al., 2020). The responsible of the production of organic acids, as acetic and gluconic acids, during fermentation are mostly AAB (Leonarski et al., 2022).

Although citric acid concentration was higher in tea infusions and kombucha compared to grape marc, the primary organic acid in grapes is tartaric acid and it was detected just in grape marc infusion and Grape-K (Table 2). Tartaric acid likely accounts for the lower pH observed in grape marc infusion (Fig. 2).

Nitrogen related compounds, PAN (primary amino nitrogen) and ammonia, decreased in their concentration because of the fermentation (Table 2). Generally, yeasts are the main consumers of nitrogen; both organic and inorganic one (Roca-Mesa et al., 2020). During wine alcoholic fermentation, ammonium and amino acids are consumed by all strains, with the same mechanisms controlling their consumption. Here, we observed a higher decrease in PAN, which indeed was depleted, rather than ammonia. This PAN preference is similar to what is observed for wine yeast during alcoholic fermentation (Crépin et al., 2014). The lower limit of nitrogen to finish the alcoholic fermentation in wine is 140 mg/L (Bely et al., 1990) but is dependent on the yeast strain and sugar concentration. Herbal products with tea potential are not considered a good nitrogen source because it does not contain purine derivatives. However, the grape marc brew had even less nitrogen than the tea brews. Despite that, ammonia was not completely depleted in any of the kombucha prepared indicating that nitrogen was not limiting (Table 2).

Polyphenolic composition presented large differences between all the samples (Table 2). Tea kombuchas presented the highest values in this parameter, mainly in black tea kombucha. These values were similar in the non-fermented and fermented tea kombuchas with no significant changes observed before and after fermentation (Table 2). The lowest values were obtained in the grape marc kombucha. This was expected as white grape varieties are not as rich in phenolic compounds as red varieties or even tea (Astill et al., 2001). In this way, black tea kombuchas showed the highest values for phenolic compounds. Besides, the concentration of TPC could be enhanced with larger fermentation duration, since the grape marc starter obtained in the first experiment had about 500 mg GAE/L (Suppl. Table 1). Anthocyanins concentration was specifically measured (Table 2). Anthocyanins are the main polyphenolics in red grape varieties, whereas flavan-3-ols are more abundant in white ones (Cantos et al., 2002). In general, anthocyanin values were low,

supported by the color of the kombuchas which appeared to be not very intense (Fig. 1).

The quantified parameters allowed to determine some key elements to characterize the changes in kombucha due to fermentation. Even if the raw material was different, the fermentative pattern was similar in all cases. The observed initial heterogeneity was generally maintained after the fermentation. Overall, the main differences were observed in the sugar content and consumption patterns and the phenolic composition.

3.3. Antioxidant capacity

Antioxidant activity is one of the most interesting and studied properties of kombucha because of its potential to prevent several diseases (Morales et al., 2023; Villarreal-Soto et al., 2019). The free radical DPPH was used to evaluate the antioxidant activity of the grape marc kombucha and compared it to the traditional tea kombucha. The three obtained kombuchas were diluted – in series of 2-fold dilutions – and evaluated the scavenging activity on a fixed concentration of DPPH ethanolic solution (Fig. 3). The obtained results were then used to adjust a sigmoidal curve for each non-fermented and fermented kombucha, which was used to interpolate the IC₅₀. Constants for the sigmoidal curves obtained can be found in Suppl. Table 3.

As reported in literature, when comparing the scavenging activity of kombucha in a fixed DPPH concentration, its inhibition activity follows a sigmoidal curve. Increasing concentrations of kombucha in the reaction medium increase the antioxidant activity observed until reaching a maximum (Fig. 3). Besides, when low concentration of kombucha is added to the reaction, no antioxidant activity is observed, until the threshold is met.

Tea kombuchas are known to have high antioxidant capacity, due to their high concentrations of phenolic compounds (Cardoso et al., 2020). It is described that the antioxidant capacity of kombuchas increases after fermentation (Villarreal-Soto et al., 2018). Here, the antioxidant capacity obtained for the tea kombuchas did not increase significantly after fermentation (Table 2). In contrast, grape marc kombucha's antioxidant capacity increased significantly after fermentation (Fig. 3). The increase in the antioxidant capacity was not related to the concentration

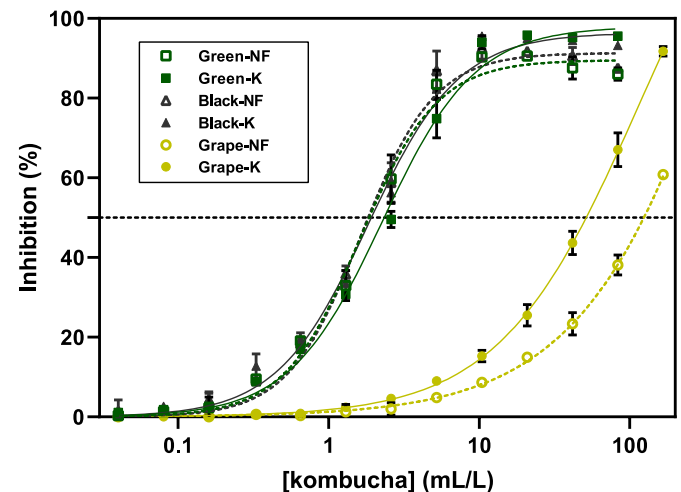


Fig. 3. The antioxidant capacity of the produced kombuchas with respect to their initial state before fermentation. Symbols represent the antioxidant inhibition potential of each kombucha concentration; empty dots and filled dots are non-fermented and after kombucha fermentation, respectively. Dotted lines and continuous lines represent the adjusted sigmoidal curve to each kombucha, respectively. Black horizontal dotted line points the IC₅₀ value. Green, black and grape means green tea, black tea, and grape marc, respectively. NF: non fermented; K: kombucha. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

of phenolic compounds, as no increase was observed from Grape-NF to Grape-K (Table 2). One possible explanation might be that phenolic compounds can undergo structural changes, leading to the formation of new compounds with enhanced antioxidant properties (Lingua et al., 2016; Rodríguez et al., 2008). In addition, the combination of compounds might interact synergistically to enhance the antioxidant activity of the individual compounds. These interactions can involve phenolics, organic acids, and other metabolites that work together to stabilize free radicals or prevent oxidative processes (Cimino et al., 2007). Finally, the microorganisms involved in the fermentation can produce antioxidants, such as glutathione, which have antioxidant properties (Shahidi & Ambigaipalan, 2015).

These results can be compared with the calculated values of IC_{50} (Table 2). For tea, the IC_{50} value was similar, from tea non-fermented to kombuchas. Besides, the IC_{50} value of 124.02 mL/L of Grape-NF, the fermented Grape-K was reduced to 51.32 mL/L. This means that to achieve 50 % of scavenging activity, it is just necessary to less the half of the volume of Grape-K regarding to Grape-NF.

Since different antioxidant activity method measurements are proposed in the literature, it is difficult to really compare these results with other works. Still, most of previous studies have reported an increase in the antioxidant capacity after kombucha-like fermentation with different raw materials, usually with long fermentation times (Morales et al., 2023). Nevertheless, under the studied conditions, and 7 days of fermentation, it was not observed this phenomenon, probably related to a very high initial antioxidant activity of the infusions (Fig. 3, Table 2).

Considering all these, even if the antioxidant capacity of the produced grape marc kombucha is less than the observed with the tea kombuchas, the fermentation process of grape marc kombucha enhances this important kombucha property.

3.4. Sensory analysis

One month after the production, a sensory analysis was organized to evaluate the characteristics, acceptance, preference, and overall quality of the kombuchas.

Before a complete descriptive analysis, the 15 panelists were asked to perform a triangular test of the three kombuchas in three series. Results showed that tasters were able to distinguish the three kombuchas between them just by smelling them in a dark glass. The series Grape-K vs both Green-K or Black-K had 13 and 12 correct assignments out of 15, demonstrating that these kombuchas were significantly different at a p -value < 0.005 . Besides, no statistically significant difference was observed when the trial was between the two tea kombuchas, with 9 correct assignments out of 15 ($p < 0.5$).

Together with the triangular tests, tasters were asked to write down their preferences. Interestingly, the Grape-K was always the preferred kombucha (92 %), always from the tests where kombuchas were correctly assessed. Besides, when comparing Green-K and Black-K, 100 % of the tasters preferred the Green-K.

After demonstrating tasters were able to significantly distinguish between the smells of the three produced kombuchas, they were asked to evaluate some selected organoleptic attributes. The summary of this information can be found in Fig. 4, where the mean of each attribute's scores is presented, and in Table 3, in which the results of the ANOVA test are collected.

It is worth noting that all the attributes studied showed significant differences between samples (Table 3), except for the aromatic intensity (Fig. 4).

First, the visual attributes of the tasted kombuchas were different. The color intensity had an increasing value from Grape-K, the lightest kombucha; to Black-K the most colorful one. The low polyphenolic concentration shown in Grape-K (Table 3) was also noticeable for the tasters and was translated in the lowest score of the color intensity attribute (Fig. 4, Table 3). On the contrary, Grape-K was the most transparent kombucha, being the Black-K the one that presented the

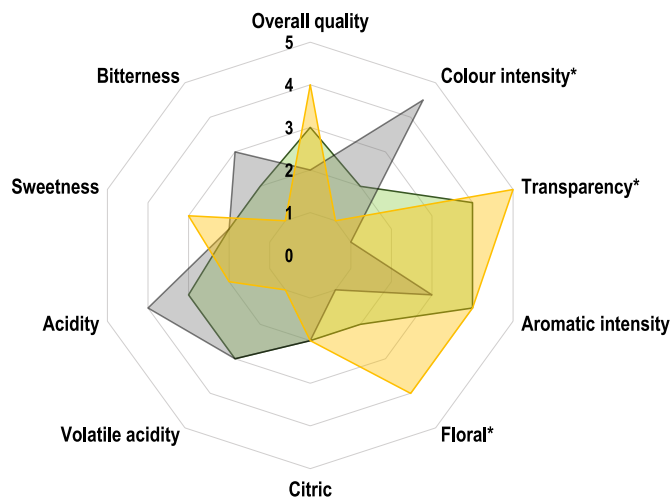


Fig. 4. Spider plot of organoleptic attributes analyzed in descriptive sensory analysis. Green, black and yellow shadowed surfaces are green tea kombucha, black tea kombucha and grape skin kombucha, respectively. Asterisks (*) indicate significant differences between all three kombuchas, p -value < 0.05 . (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

highest turbidity. The increased turbidity in Black-K can be attributed to a higher concentration of polyphenols and other compounds that cause colloidal instability, leading to a less transparent beverage. The transparency of Grape-K suggests lower levels of suspended solids and colloids, which is consistent with its lower phenolic content.

Second, the aromatic intensity of the three kombuchas was similar (Fig. 4). Still, some differences were observed (Table 3). Grape-K was produced with grape marc from the Muscat of Alexandria variety, which is known to have enhanced concentrations of floral notes (Bordiga et al., 2013), had the highest floral attribute score. Indeed, the use of this grape variety was not arbitrary. It was used to produce a kombucha-like beverage with a new aromatic profile, which the tasters perceived positively. The citric aroma, which is also related to tea kombuchas, was more noticeable in Green-K rather than in Black-K, with the Grape-K in between. The most interesting result for the aromatic attribute was found in the volatile acidity perception. Grape-K had the lowest perception in this attribute (Table 3), having the highest acetic acid concentration (Table 2). It seems that the enhanced floral aroma has counteracted the volatile acidity perception in Grape-K. This phenomenon is supported by sensory studies indicating that strong aromatic profiles can diminish the perception of sourness and acidity in beverages. This result highlights the complex interplay between different sensory attributes in determining overall flavor perception (Bayarri et al., 2001).

Third, according to gustative attributes, Grape-K was the one that presented the lowest scores for acidity and bitterness. The acidity perception follows the results obtained at the end of the fermentation (day 7), where Grape-K presented the highest value for pH (Fig. 2). The bitterness, which can be usually reported as an unpleasant gustative attribute, when unbalanced and excessive, was found to be less present in Grape-K.

To finish, the overall quality of the kombuchas was ranked with an average score of 5 for Grape-K, 4 for Green-K, and, finally, 3 for Black-K (Fig. 4). Still, Green-K and Grape-K had no significant differences in this attribute, being significantly lower than those obtained in Black-K (Table 3).

The acceptance of the newly produced Grape-K was demonstrated after the sensory analysis in which (i) tasters were able to distinguish the raw material of the produced kombuchas, (ii) most of them significantly preferred Grape-K, and (iii) unpleasant attributes' scores as volatile acidity and bitterness were the lowest.

Table 3Results of the Tukey *post hoc* test of the one-way ANOVA ($p < 0.05$) for scores of attributes of the three tested kombuchas in the descriptive sensory analysis.

	Colour intensity	Transparency	Floral	Citric	Volatile acidity	Acidity	Sweetness	Bitterness	Overall quality
Green	B	B	B	B	B	B	AB	AB	B
Black	C	A	A	A	B	B	A	B	A
Grape	A	C	C	AB	A	A	B	A	B

4. Conclusions

Currently, the use of by-products is not only a trend in winemaking but also a need for suitable development in food science. Here, we reused a common white winemaking by-product to produce a new kombucha-style beverage.

The fermentation process of both tea and grape marc kombucha was similar in terms of acid production, but the Grape-K showed enhanced sugar consumption. Interestingly, the obtained SCOBY from grape marc kombucha fermentation showed similar microbial composition no matter the raw fermentative material. The microbial population was found to be dominated by *Brettanomyces bruxellensis* (yeast), *Oenococcus oeni* and *Liquorilactobacillus ghanensis* (lactic acid bacteria), and *Komagataeibacter intermedius* (acetic acid bacteria).

The chemical composition of the obtained kombuchas was similar with lower concentrations of phenolic compounds and higher acetic acid in Grape-K. Even if grape marc kombucha showed less antioxidant activity than tea kombuchas, this was significantly increased after seven days of fermentation. Besides, the use of Muscat grape marcs was shown to be interesting to improve the floral notes of the kombucha and to produce an attractive product for customers. Indeed, the volatile acidity perception seems to be counteracted by the other aromas found in Grape-K.

Altogether, grape marc, mainly from white aromatic varieties, seems to be an interesting raw material for producing kombuchas with differentiated aroma attributes.

Future studies could investigate the antimicrobial activity of kombucha against various pathogens, explore correlations between microbial communities in the SCOBY and the organoleptic properties of kombucha, and analyze volatile organic compounds to identify key molecules that contribute to flavor and aroma.

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CRediT authorship contribution statement

Aitor Balmaseda: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation. **Estefania Romeu:** Writing – original draft, Methodology, Investigation, Data curation. **Albert Mas:** Writing – review & editing, Resources, Project administration, Funding acquisition. **Maria C. Portillo:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.lwt.2024.116882>.

Data availability

Data are included in the manuscript

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